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A Soluble Form of Phosphatase in *Saccharomyces cerevisiae* Capable of Converting Farnesyl Diphosphate Into *E,E*-Farnesol

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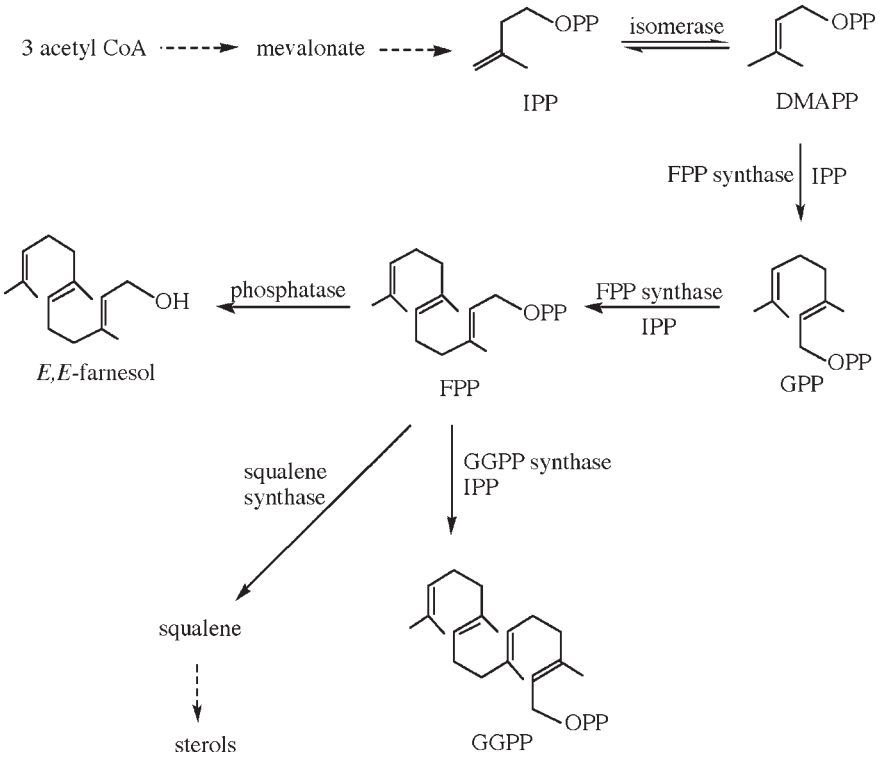
Abstract

After anion-exchange chromatography, the soluble fraction of a cell-free extract of *Saccharomyces cerevisiae* showed two phosphatase activity peaks when *p*-nitrophenyl phosphate (pNPP) was used as the substrate. However, only the second pNPP active peak demonstrated the ability to convert farnesyl diphosphate (FPP) into *E,E*-farnesol. N-terminal sequence analysis of the purified pNPP/FPP phosphatase revealed that it was a truncated form of alkaline phosphatase Pho8 lacking 62 amino acids from the N-terminus and was designated Pho8Δ62. Although other isoprenyl diphosphates such as geranyl diphosphate (GPP) and geranylgeranyl diphosphate (GGPP) could also be hydrolyzed by Pho8Δ62 to the corresponding alcohols, selectivity was observed among these substrates. The optimum pH was 7.0 for all three isoprenyl diphosphate substrates. Although lower hydrolytic activity was observed for FPP and GGPP at pH 6.0 and 8.5, hydrolysis of GPP was observed only at pH 7.0. Mg^{2+} and Mn^{2+} inhibited hydrolysis of FPP and GGPP, and GGPP was more sensitive to Mg^{2+} inhibition than FPP. The rate of FPP hydrolysis increased in the presence of Triton X-100.

Index Entries: Phosphatase; farnesyl diphosphate; isoprenoid; farnesol.

Introduction

All living systems require the isoprenoid pathway to synthesize a wide array of products such as carotenoids, ubiquinones, dolichols, sterols, terpenes, and prenylated proteins for various cellular functions. There is great interest in the metabolic engineering of this pathway to produce



Scheme 1. Biosynthetic pathway of isoprenoids. Dashed arrows indicate multisteps. OPP, diphosphate.

isoprenoids of commercial value *(1,2)*. *Saccharomyces cerevisiae* employs three molecules of acetyl coenzyme A to construct isopentenyl diphosphate (IPP), which is further converted into dimethylallyl diphosphate (DMAPP) by an isomerase (Scheme 1). Two molecules of IPP are sequentially condensed with DMAPP by farnesyl diphosphate (FPP) synthase to form geranyl diphosphate (GPP) and then FPP. Further chain elongation by the addition of another IPP to FPP leads to geranylgeranyl diphosphate (GGPP). Two molecules of FPP are condensed by squalene synthase to form squalene from which sterols are derived. Although wild-type (WT) yeast *S. cerevisiae* has no detectable FPP or farnesol accumulation, mutants blocked at squalene synthase accumulated FPP intracellularly *(3)* and *E,E*-farnesol in the culture medium *(2)*. The intracellular accumulation of FPP in the yeast mutants points to the importance of identifying and overexpressing a soluble phosphatase capable of converting FPP into *E,E*-farnesol in order to increase farnesol production further.

This article describes the identification and properties of the only detectable phosphatase in the soluble cell-free extract of *S. cerevisiae* that is capable of converting FPP into *E,E*-farnesol.

Materials and Methods

Reagents

Triammonium salts of GPP, FPP, and GGPP as well as geraniol, *E,E*-farnesol, geranylgeraniol, and pNPP were from Sigma (St. Louis, MO). Protein was measured using a Bio-Rad Protein Assay kit with IgG as the standard. Anion-exchanger Macro-Prep High Q was from Bio-Rad (Hercules, CA) and L-histidyldiazobenzyl phosphonic acid on agarose was from Sigma. All protein purification work was performed at 4°C.

Gas Chromatography-Mass Spectrometry Measurement of Isoprenyl Alcohols

The detection of geraniol, farnesol, and geranylgeraniol was performed using a Hewlett Packard gas chromatograph equipped with a mass selective detector using an Rtx-5MS column (Restek, 0.25 mm id, 15 m, 1 μm). The temperature of the oven was initially held at 80°C for 1 min, followed by an increase to 300 °C at 15 °C/min. To reduce run time, the initial temperature of the oven was set at 100°C for the detection of farnesol and geranygeraniol. Helium was the carrier gas and the column head pressure was at 13 psi. Both the injector and detector were at 300°C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Protein was analyzed using 4–12% NUPAGE Bis-Tris gels with MOPS running buffer at 200 V and room temperature. All buffers, precast gels, and stains were from NOVEX (now Invitrogen).

Phosphatase Assays Using p*-Nitrophenyl Phosphate as Substrate*

The assay was performed in a 0.1-mL mixture containing 15 m*M* Bis-Tris propane (BTP)/HCl, pH 7.0; 10 m*M* $MgCl_2$; 5 m*M* *p*-nitrophenyl phosphate (pNPP); and an enzyme source by incubating at 30 °C for 15 min. Absorption at 400 nm was measured after the addition of 0.8 mL of BTP buffer.

Assay of Partially Purified Phosphatase Using FPP as Substrate

A 0.1-mL mixture containing 50 m*M* BTP/HCl, pH 7.0; 5 m*M* $MgCl_2$; 5 μg of FPP; and crude enzyme was incubated at 30°C for 30 min. Methanol (0.05 mL) was added and the mixture was extracted with 0.3 mL of hexane. The amount of *E,E*-farnesol was determined using gas chromatography-mass spectrometry (GC-MS).

Assay of Purified Phosphatase Using FPP as Substrate

The purified phosphatase was assayed in a 0.1-mL mixture containing 5 μg of FPP; 0.1 *M* BTP/HCl, pH 7.0; and the enzyme by incubating at 30°C for 20 min, followed by extraction with 0.2 mL of hexane. No magnesium

was added, owing to its inhibitory effect against the purified enzyme. Farnesol was measured using GC-MS.

N-terminal Sequence Analysis

Sequencing was performed at the PAN Facility of Beckman Center at Stanford University Medical Center, Palo Alto, CA.

Soluble Cell-Free Protein Extract

WT yeast *S. cerevisiae* (ATCC 28383) was cultured in YPD (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of glucose) at 30°C for 24 h starting at OD_{600} = 0.1. Cells were pelleted at 10,000*g*, washed twice with water, and stored at –80°C. A suspension of 6 g of wet pellet in 18 mL of 15 m*M* BTP/HCl, pH 7.0, containing 20% (v/v) glycerol was homogenized twice in an ice-chilled French press cell at 20,000 psi. The homogenate was centrifuged at 146,000*g* for 1 h. The supernatant was filtered using a 0.45-µm polyvinylidene fluoride filter.

Purification of Protein

The extract obtained was applied to a Macro-Prep High Q anion-exchange column (1.6 × 12 cm) at 2 mL/min. The column was washed with 30 mL of buffer A (15 m*M* BTP/HCl, pH 7.0) at 3 mL/min and eluted with a gradient of 0–50% buffer B (A + 1 *M* NaCl) over a volume of 250 mL. Fractions of 6 mL were collected.

Fractions in the second pNPP active peak that appeared during the NaCl gradient elution of the anion-exchange column were combined, and the buffer was changed to 15 m*M* BTP/HCl, pH 7.0, by repeated concentration and dilution using a 10-kDa Ultrafree-15 centrifugal device (Millipore, Bedford, MA). The sample was applied onto a column of L-histidyldiazobenzyl phosphonic acid on agarose (1 × 8 cm bed) at 0.5 mL/min. The column was washed at 1 mL/min with 50 mL of buffer A (15 m*M* BTP/HCl, pH 7.0), followed by 30 mL of buffer B (A + 1 *M* NaCl). The enzyme was eluted with 30 mL of buffer C (A + 20 m*M* potassium phosphate).

Results

Purification and Identification of Phosphatase Capable of Hydrolyzing FPP to Farnesol

The soluble fraction of a cell-free extract of WT *S. cerevisiae* was fractionated using anion-exchanger Macro-Prep High Q. Two peaks of phosphatase activity utilizing pNPP as the substrate were detected during the NaCl gradient elution (Fig. 1). When the fractions were examined for the phosphatase activity of converting FPP into *E*,*E*-farnesol, only the second pNPP hydrolyzing peak was active. Higher FPP hydrolysis activity was observed in the presence of 5 m*M* $MgCl_2$ compared to assays without added magnesium. This pNPP/FPP dual activity was able to bind to the phosphonic

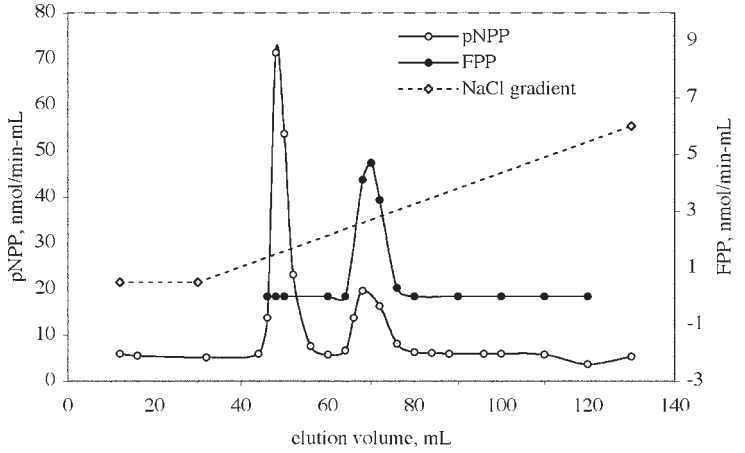


Fig. 1. Distribution of phosphatase activity in fractions after anion-exchange chromatography using Macro-Prep High Q. Buffer A (15 m*M* BTP/HCl, pH 7.0) was used to elute from 0 to 30 mL followed by an NaCl gradient at 0.2% buffer B (A + 1 *M* NaCl)/mL. The activity of FPP hydrolysis was assayed in the presence of 5 m*M* $MgCl_2$.

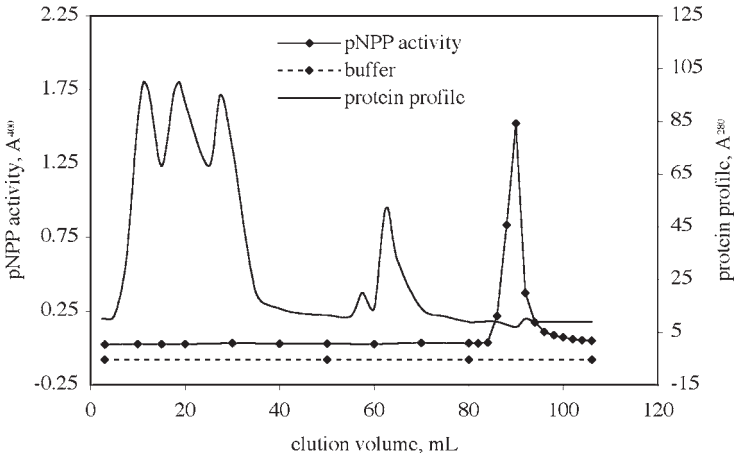


Fig. 2. Protein profile and distribution of phosphatase activity in fractions after phosphonic acid column. Step elution included buffer A (15 m*M* BTP/HCl, pH 7.0) to 50-mL elution and buffer B (A + 1 *M* NaCl) up to 80-mL total elution followed by buffer C (A + 20 m*M* K-PO_4, pH 7.0). The substrate was pNPP, and the starting material was the combined fractions capable of hydrolyzing FPP in Fig. 1 after buffer exchange.

acid column even in the presence of 1 *M* NaCl. Elution of the phosphonic acid column using a buffer containing 10 or 20 m*M* potassium phosphate showed a single phosphatase peak that could utilize both pNPP and FPP (Fig. 2). Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a protein band at about 60 kDa after silver staining. The N-terminal sequence was determined to be SHKKKNVIFFVTDGMGP, and it completely matched the sequence of yeast alkaline phosphatase encoded by

Table 1
Effect of pH on Hydrolysis of GPP, FPP, and GGPP Catalyzed by Pho8Δ62[a]

pH	GPP to GOH (μmol/[min·mg])	FPP to FOH (μmol/[min·mg])	GGPP to GGOH (μmol/[min·mg])
6.0	0 (0%)	0.049 (0.8%)	0.33 (30%)
7.0	3.2 (100%)	6.4 (100%)	1.1 (100%)
8.5	0 (0%)	0.52 (8%)	0.69 (63%)

[a]All reactions were carried out at 30°C for 20 min. Substrates were examined individually under the following conditions: [GPP] = 109 μ*M*, [enzyme] = 0.4 μg/mL; [FPP] = 115 μ*M*, [enzyme] = 0.6 μg/mL; [GGPP] = 99.7 μ*M*, [enzyme] = 3 μg/mL. Relative activities to pH 7.0 for each substrate are shown in parentheses.

GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol.

PHO8 from positions 63 to 79. Therefore, the purified phosphatase was a truncated Pho8 lacking 62 amino acids at the N-terminus and was designated Pho8Δ62. The molecular mass for the 62 lost amino acids is 6770 Daltons.

Properties of Purified Pho8Δ62

Effect of pH

The effect of pH was investigated by the individual hydrolysis of GPP, FPP, and GGPP at pH 6.0 (0.1 *M* Bis-Tris/HCl), 7.0 (0.1 *M* BTP/HCl), and 8.5 (0.1 *M* BTP/HCl). Reactions were carried out at 30°C for 20 min in a total volume of 0.1 mL. The isoprenyl alcohol products were extracted into 0.1 mL of hexane and measured using GC-MS. The enzyme Pho8Δ62 showed optimum activity at pH 7.0 for all three isoprenyl diphosphate substrates (Table 1). GPP appeared to be the most sensitive to changes in pH and GGPP was the least sensitive. An individual control experiment containing one of the substrates showed no formation of alcohol in the absence of the enzyme, indicating that the hydrolysis of the isoprenyl diphosphate was owing to the action of the phosphatase.

Effect of Metal Ions, Phosphate, and Triton X-100

All reactions investigating the effect of metal ions and phosphate were carried out using FPP or GGPP as the substrate at pH 7.0 and 30°C for 20 min. Phosphate was added in the form of potassium salt. Phosphate, $MgCl_2$, and $MnCl_2$ showed inhibition toward the hydrolysis of both FPP and GGPP at the concentrations tested (Table 2). Copper showed no inhibition at a concentration of 2 m*M*. Whereas $CaCl_2$ and $ZnSO_4$ showed no inhibition against the hydrolysis of FPP, they both inhibited the hydrolysis of GGPP.

The effect of magnesium on the hydrolysis of FPP and GGPP was further investigated at lower magnesium concentrations and pH 7.0. It was found that the hydrolysis of GGPP was more sensitive to magnesium inhibition. At a concentration of 0.06 m*M*, Mg^{2+} showed no inhibition toward the hydrolysis of FPP whereas 60% inhibition was observed against the hydrolysis of GGPP (Fig. 3). The presence of 0.05 g/L of Triton X-100 showed increased FPP hydrolysis (Table 3).

Table 2
Effect of Divalent Metal Ions and Phosphate
on Hydrolysis of FPP and GGPP Catalyzed by Pho8Δ62[a]

	None	2 m*M* $MgCl_2$	2 m*M* $MnCl_2$	2 m*M* $CaCl_2$	2 m*M* $ZnSO_4$	2 m*M* $CuSO_4$	$K\text{-}PO_4$
FPP	1.0	0.078	0.070	1.1	1.4	1.0	0.15
GGPP	1.0	0	0	0.33	0.53	1.3	0.21

[a]Effect was compared based on relative activity for each substrate. Substrates were examined individually under the following conditions: [FPP] = 46.1 μ*M*, [enzyme] = 0.4 μg/mL, [$K\text{-}PO_4$] = 1 m*M*; [GGPP] = 59.8 μ*M*, [enzyme] = 1 μg/mL, [$K\text{-}PO_4$] = 2 m*M*.

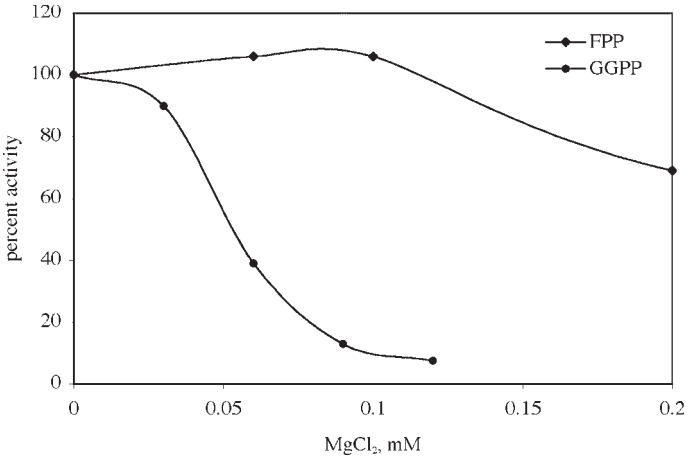


Fig. 3. Effect of $MgCl_2$ on hydrolysis of FPP and GGPP catalyzed by Pho8Δ62 at pH 7.0. Conditions: [FPP] = 46.1 μ*M*, [Pho8Δ62] = 0.4 μg/mL; [GGPP] = 59.8 μ*M*, [Pho8Δ62] = 1 μg/mL. The activity for each substrate was normalized individually.

Table 3
Effect of Triton X-100 on Hydrolysis of FPP Catalyzed by Pho8Δ62[a]

FPP (μ*M*)	No Triton X-100 added	With 0.05 g/L of Triton X-100
46	1.83	4.22
69	2.32	6.32
115	1.79	6.96

[a]All reactions were at pH 7.0 and contained 0.4 μg/mL of enzyme. The rate of hydrolysis is expressed in micromoles per minute per milligram.

Discussion

Although anion-exchange chromatography of the soluble fraction of a cell-free extract of *S. cerevisiae* showed two phosphatase activity peaks utilizing pNPP, only the second peak was found capable of converting FPP into farnesol. This phosphatase was purified, and its N-terminal sequence

of 17 amino acids indicated that it was a truncated form of alkaline phosphatase Pho8 lacking 62 amino acids from the N-terminus. Two specific phosphatases, FPPase hydrolyzing FPP to farnesol and GGPPase hydrolyzing GGPP to geranylgeraniol, were previously reported in rat liver microsomes *(4)*. Another FPPase was identified in Chinese hamster ovary cells *(5)*. In contrast to those specific phosphatases, the truncated yeast phosphatase Pho8Δ62 identified here showed no specificity for the complete dephosphorylation of GPP, FPP, and GGPP. Unlike the previous FPPase and GGPPase that showed acidic pH optima, the yeast Pho8Δ62 showed optimum activity at pH 7.0. Although nonspecific for GPP, FPP, and GGPP, Pho8Δ62 showed some degree of selectivity toward these substrates under various conditions. For example, the hydrolysis of GPP by Pho8Δ62 is the most sensitive to changes in pH and GGPP is the least sensitive. The selectivity of Pho8Δ62 is also reflected by the effect of divalent metal ions. Although Mg^{2+} is inhibitory for the hydrolysis of FPP and GGPP, the hydrolysis of GGPP by Pho8Δ62 is more sensitive to Mg^{2+} inhibition. At a concentration of 0.06 m*M*, Mg^{2+} had no effect on FPP hydrolysis but showed 62% inhibition toward the hydrolysis of GGPP. In contrast to the purified enzyme, the addition of magnesium ion to assays using the crude enzyme showed increased hydrolysis of FPP. As a result, different assay conditions were employed. Another difference is that Ca^{2+} and Zn^{2+} had no negative effect on FPP but both inhibited GGPP hydrolysis.

Native alkaline phosphatase Pho8 is a glycoprotein localized to the yeast vacuole *(6)*. It is synthesized as an inactive precursor and anchored in the membrane by a hydrophobic N-terminal domain with the C-terminus inside the vacuole. Protease A activates this phosphatase by removing a small peptide from the C-terminus. The *PHO8* gene has been cloned and the enzyme is composed of two 66-kDa subunits, about 8% of which is carbohydrate *(7)*. It was later purified and shown to be fructose-2,6-bisphosphate 6-phosphatase *(8)*. A truncated form of Pho8 lacking 60 amino acids from the N-terminus including the membrane-spanning region was previously constructed and shown to be in the cytosol *(9)*. It will be interesting to overexpress Pho8Δ62 and observe its effect on the accumulation of FPP and farnesol in yeast mutants blocked at squalene synthase.

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